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RESEARCH ARTICLE

L-Ascorbic Acid (LAA) Supplementation Enhances Proliferation and Reduces Cellular Senescence in Adipose-Tissue Mesenchymal Stem Cells (AT-MSC) Without Altering Characterization

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Abstract

Adipose-tissue mesenchymal stem cells (AT-MSCs) and their secretome have been widely utilized in the field of anti-aging and regenerative medicine. However, the number of AT-MSCs declines during subculture due to cellular senescence. The addition of antioxidant such as L-ascorbic acid (LAA), which has been shown to promote proliferation and differentiation while reducing oxidative stress, may help mitigate cellular senescence. Nevertheless, the optimal dose of LAA supplementation for AT- MSC culture remains unclear. To identify the potential optimal dose, a cell survival assay was performed. Following the determination of the optimal dose, the morphology, proliferation, viability, differentiation, and characterization of AT-MSCs were evaluated. Additionally, a senescence-associated β-galactosidase (SA- β-Gal) assay was conducted to assess the effect of LAA on cellular aging.

LAA at concentration of 100 and 200 µg/mL LAA demonstrated the ability to maintain high cell viability. Proliferation of AT-MSCs increased significantly in a dose-dependent manner ($p < 0.05$) following LAA supplementation, while cell viability remained above 90% ($p > 0.05$), indicating no significant cytotoxicity. LAA treated AT-MSCs also successfully differentiated into chondrocytes, osteocytes and adipocytes, comparable to those cultured under standard conditions. Throughout serial passages, AT-MSCs consistently expressed CD90, CD73, with lower expression of CD105. Moreover, LAA treatment at 100 µg/mL significantly reduced ($p < 0.05$) the number of senescent cells. Both 100 and 200 µg/mL doses were effective in maintaining cell morphology, supporting high viability, enhancing proliferation, and preserving the differentiation potential. These doses also contributed to reducing cellular senescence while maintaining the expression profile of MSCs surface marker across passages.

Keywords: AT-MSCs, L-ascorbic acid, Proliferation, Viability, Differentiation, Characterization, Senescent cell

Introduction

Stem cells possess remarkable proliferative potential and an extraordinary capacity to differentiate into diverse cellular lineages, making them a captivating focus of research in anti-aging studies.[1] Mesenchymal stem cells (MSCs), in particular, are known for their high proliferative ability, especially when derived from young donor. In addition, MSCs exert anti-aging effects through their secretion of cytokines and growth factors that promote angiogenesis, reduce inflammation, and inhibit apoptosis.[2] MSCs can be isolated from various tissues, with bone marrow-derived MSCs (BM- MSCs) and AT-MSCs being among the most extensively studied.

AT-MSCs have several advantages, such as the higher yields of AT-MSCs can easily be obtained from

subcutaneous region through a minimal invasive and painless procedure, also be able to maintain their phenotype longer in culture, showed a greater proliferative capacity, [3] and may be more suitable for allogeneic transplantation [4]. Furthermore, AT-MSCs can be differentiate into many cell types including adipocytes, osteoblasts, chondrocytes, neurocytes, and hepatocytes [5]. Even it was due those advantages of AT-MSCs, however in terms of cell culture, the source and repeated passsaging of MSCs during propagation can lead to cellular aging, characterized by increased cell size, reduced proliferation, impaired function, altered immunophenotype, and diminished therapeutic potential.[6] To mitigate cell aging during the culture period, several antioxidants like LAA can be added to the culture medium.[7-9] LAA enhances the secretion of growth factors and anti-inflammatory cytokines that play roles in homeostasis regulation and tissue regeneration [9]. Yang *et al*. [7] found that LAA can delay the senescent of MSC through reactive oxygen species (ROS) and protein kinase B/mammalian target of rapamycin (AKT/mTOR) signaling. The standard dose of LAA to suppress aging in MSC cultures remains unknown, necessitating further research and optimization. This study aims to determine the optimal dose of L-ascorbic acid (LAA) that support high cell yield while preserving mesenchymal stem cell (MSC) characteristics during propagation and mitigating cellular senescence.

Materials and methods

Ethical considerations

The source of the cells from lipoaspirates of patient in Plasthetic Clinic, Bendungan Hilir Tanah Abang, Jakarta, Indonesia. Ethical approval of the experiment objects that used in this study by the Ethics Committee of Faculty of Medicine Atma Jaya Catholic University of Indonesia on the use of patient wasted fat tissue, in the number 17/06/KEP-FKIKUAJ/2023, date of June 19th 2023.

Isolation and culture of AT-MSCs

AT-MSCs were isolated from subcutaneous lipoaspirate obtained from a patient undergoing abdominal liposuction. Following extraction, a transport medium consisting of low-glucose (100 mg/dL) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mM L-glutamine and 1% antibiotic-antimycotic solution (penicillin 10,000 units/mL, streptomycin 10,000 mg/mL, and amphotericin B 25 mg/mL) was added to the tube containing the lipoaspirate (adipose tissue mixed with tumescent fluid). The tube was then placed in a cool box with ice packs, maintaining a temperature of 8 - 20 °C, and transported to the laboratory for further processing. Isolation of AT-MSCs was performed following the protocol described by Karina *et al.* [6]. Initially, the adipose tissue was separated from the tumescent fluid using a sterile coffee filter and washed with 1X phosphate-buffered saline (PBS, pH 7.4) until free of blood. The washed tissue was then transferred into a sterile 50 mL centrifuge tube, mixed with a 0.075% collagenase type I solution (Sigma, USA), and incubated at 37 °C in a CO_2 incubator for 1 h with agitation every 5 min.

After incubation, the liquid phase (infranatant) was removed using a serological pipette and transferred into a sterile 15 mL centrifuge tube, followed by centrifugation at 1,200 rpm for 10 min. The supernatant was discarded using a serological pipette, leaving only the cell pellet, referred to as the stromal vascular fraction (SVF), at the bottom of the tube. The SVF was then resuspended in complete culture medium consisting of low-glucose DMEM supplemented with 4 mM L-glutamine, 10% human serum (HS), and 1% sterile antibiotic-antimycotic solution, filtered through a 0.2 µm membrane filter. The cell suspension was seeded into 12-well culture plates. The initial seeding density was standardized for each group: 375×10^3 cells/well for passage 4 (P4) and 125×10^3 cells/well for passage 7 (P7), each in triplicate. The cultures were incubated at 37 °C in a humidified environment containing 5% CO_2.

After 2 to 3 days of culture, the cells were observed, and the culture medium was replaced every 2 - 3 days. The cells were monitored until fibroblast-like, plastic adherent cells were visible at the bottom of the 12-well culture plates. Once the cells reached 70% - 80% confluence, they were harvested; by first discarding the culture medium and rinsing the wells twice with 1X PBS (pH 7.4) The TripLETM Select Enzyme (Thermo- Fisher Scientific, USA) was then added to the wells to detach the cells. Cell detachment was monitored under an inverted microscope. Once

complete detachment was observed, complete culture medium was added to neutralize the enzymatic reaction. The cell suspension was transferred into a sterile 15 mL centrifuge tube and centrifuged at 1,200 rpm for 10 min, The resulting pellet was washed with sterile 1X PBS (pH 7.4) and centrifuged again at 1,200 rpm for 10 min. This washing step was repeated twice. Finally, the cells pellet was resuspended in complete culture medium for expansion or further subculture (passaging).

3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The aim of this assay was to determine the safe dose range of LAA that does not induce toxic effects on AT-MSCs. AT-MSCs at passage 3 (P3) from each donor, having reached 70% - 80% confluency, were harvested and rinsed twice with 1X PBS (pH 7.4). The MTT assay was conducted as follow: First, the cultured medium consisting DMEM supplemented with 10% HS and antibiotic - antimycotic mixed stock medium (ABAM) and DMEM) was prepared. LAA stock solution (25 mg) was diluted in the supplemented medium to obtain concentration ranging from 0 to 500 ug/mL, which were prepared in separated tubes. Next, the cells were seeded in 96-well culture plates at the density 3,750 cells per well, in triplicate, and incubated overnight at 37 °C in a humidified environment containing 5% CO_2. After incubation, the cells were washed with 1× PBS, and the LAA dilutions were added to the corresponding wells as pre-mapped. The plates were incubated for 48 h. Following treatment, the cells were washed again with 1× PBS. Subsequently, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for additional 4 h at 37 °C in a 5% CO_2 incubator. Finally, 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and absorbance was measured at 570 nm using Multiskan Spectrum spectrophotometer (Thermo Scientific, San Jose, CA, USA). The LAA concentration that significantly increased AT-MSCs viability were selected for further evaluation, focusing on their potential to prevent AT-MSC aging during culture and determine the optimal supplementation dose.

Characterization AT-MSCs

Confluent AT-MSCs were harvested using trypsin and washed twice with PBS. The cell suspension was then transported to other laboratory in a cooler box maintained at approximately 11 - 20 °C and analyzed on the same day. The time from harvesting to analysis was approximately 1 - 2 h. For flow cytometry analysis, cells were incubated for 30 min at 4 °C with the following antibodies: anti-human-CD73-APC (Cat. No. 560847 - BD Biosciences), antihuman- CD90-FITC (Cat. No. 555595 - BD Biosciences), anti-human-CD105-PerCP (Cat. No. 560819 - BD Biosciences), and a cocktail of CD34/CD45/CD11b/CD19/HLA-DR-PEA. After staining, the cells were washed twice with PBS. A total of 10,000 cells per sample were acquired using BD Facs Lyric 8C Flow Cytometer (Becton, Dickinson and Company - BD Biosciences (BDB), USA). Cell populations were first gated using a Forward scattered cell (FSC) vs Size scattered cell (SSC) plot to identify the main cell population. A single-cell population was further isolated using a Forward scattered cell-Area (FSC-A) vs Forward scattered cell-Height (FSC-H) plot (**Figure 1**). Isotype controls were used to distinguish between positive and negative signals [9].

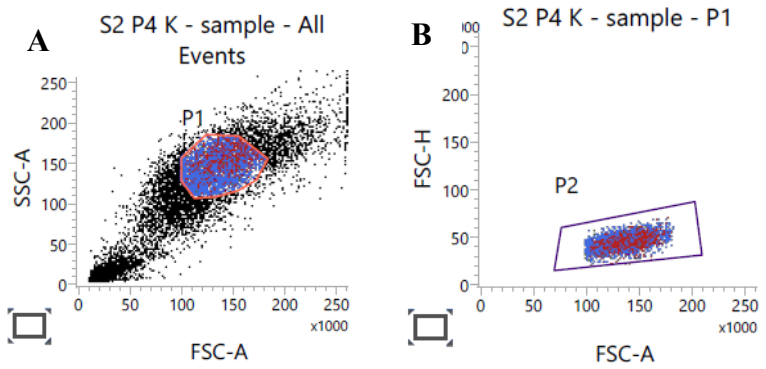

Figure 1 Gating strategy for flow cytometry analysis. (A) FSC vs SSC (B) FSC-A vs FSC-H.

Differentiation test of AT-MSCs

Passage 3 of AT-MSCs from donors in 70% - 80% confluent was harvested and washed twice with 1x PBS (pH 7.4). The cells were then resuspended in complete cultured medium (as describe in the *Isolation and Culture of AT-MSCs* section) and seeded into 24-well culture plates at the density of 20,000 cells per well, in triplicate. Once the cells reached 70% - 80% confluence, the medium was replaced with differentiation induction media for adipogenesis and osteogenesis, according to the as manufacturer's instruction (StemPro™ Adipogenesis and Osteogenesis Kits, Cat. No. A1007001 and Cat. No. A1007201; Thermo Fisher Scientific, Waltham, MA, USA). For the chondrogenic differentiation, cells were maintained in prolonged culture (over-confluent) in complete culture medium (StemPro™ Chondrogenesis Kits, Cat. No. A1007101; Thermo Fisher Scientific, Waltham, MA, USA). Cell morphology was observed using inverted microscope (Nikon Eclipse Ti). Differentiation was indicated by morphological changes after 4 days of induction. Specific staining of Oil Red O for adipocytes, Alcian Blue for chondrocytes, and Alizarin Red for osteocytes, was used to confirm cell differentiation.

Testing the potential of L-ascorbic acid in preventing aging of AT-MSCs

The next experiment aimed to evaluate the potential of LAA in preventing the aging of AT-MSCs during the culture period. This assessment was based on the finding of Liao *et al*. [10], who reported an increase in the mRNA expression of cell senescence markers p16, p21, and p53 in AT-MSCs at P7 compared to P4. To investigate the potential of LAA as a culture supplement for AT-MSCs, the evaluation included analysis of cell morphology, proliferation (based on cell number ratio), viability, and SA-β-Gal activity as an indicator of cellular senescence.

Proliferation and viability of AT-MSCs

P4 and P7 from donors in 70% - 80% confluent was harvested and washed twice with 1× PBS (pH 7.4). The cells were then resuspended in a complete cultured medium and seeded into 25 cm^2 flask at density of 375×10^3 cells per well (P4) and 125×10^3 cells per flask (P7), in triplicate for 14 days of culture. The culture medium was replaced every 2 - 3 days. On the 14^{th} day, cells were harvested and counted the alive and dead cells with trypan blue staining in a hemocytometer under an inverted microscope (Nikon Eclipse Ti). Cell ratio determined the comparison of cells after 7 days treatment against 1^{st} time treatment of cells, should have been done after counting cells, and become the options to know cell's proliferation. Cell viability was calculated with the formula:

$$Cell\ viability = \frac{Number\ of\ live\ cell}{Total\ cells} \times\ 100\%$$

Senescence-associated-beta-galactosidase (SA-β-Gal) activity test

SA-β-Gal activity test was commonly used to evaluate aging process in MSCs cultures. The activity of SA-β-Gal was detected using the Senescent Histochemical Staining Kit (#CS0030, Sigma-Aldrich, USA) according to the manufacturer's instruction:

P4 and P7 cells from donors, at 70% - 80% confluency, were harvested and washed twice with PBS 1X (pH 7.4). The cells were then resuspended in complete cultured medium and seeded into 6-well

culture plates at a density 19,000 cells per well (in triplicate). Once the cultures reached 70% - 80% confluency, the medium was aspirated, and the cells were washed twice with 1×PBS (pH 7.4). Next, 300 µL 1x fixation buffer was added to each well, and the cells were incubated at room temperature for 6 - 7 min. After incubation, the fixation buffer was aspirated, and the cells were washed 3 times with 1×PBS (pH 7.4). Then, 200 µL of staining mixture was added to each well, the wells were sealed with parafilm and incubated at 37 °C without CO_2 until the cell turned blue (approximately 2 h to overnight, depending on the required staining time). Finally, the staining mixture was removed and replaced with 1×PBS (pH 7.4). The cells were observed using an inverted microscope (Nikon Eclipse Ti) in 5 randomly selected fields. The number of blue-green stained cells (SA-β-Gal positive) in each field was counted [11]. SA-β-Gal activity was expressed as the percentage of positive relative to the total number of cells.

Statistical analysis

The results data in P4 and P7 for each parameter (replicated sample, $n = 3$) were analyze for normality and homogeneity using the Shapiro-Wilk and Levene's tests, respectively. If the data were normally distributed and homogenous, a one-way analysis of variance (ANOVA) was conducted, followed by Bonferroni post hoc analysis. If the data were not normally distributed or not homogenous, the Kruskal-Wallis's test was used for group rank comparison. Additionally, to evaluate the effect of LAA across passages, the data were analyzed using by paired Student's *t*-test or the Wilcoxon signed-rank test, as appropriate. A *p*-value of < 0.05 with a 95% confidence interval was considered statistically significant.

Results and discussion

Surviving test of LAA on AT-MSCs by MTT assay

Cells viability of AT-MSC after being treated with LAA at concentrations ranging from 0 - 500 µg/mL is shown in **Figure 2**. The MTT assay revealed a significant increase in cell viability in P3 cultures following LAA supplementation. An increase in cell viability (%) was observed starting at 100 µg/mL of LAA, with dose-dependent enhancement. ANOVA results indicated that all LAA concentrations significantly increased AT-MSC viability compared to the untreated groups ($p < 0.05$; $p < 0.01$). Post hoc analysis showed that LAA concentration of 100 and 200 µg/mL were significantly different ($p < 0.01$) from the other treated group. As also shown in **Figure 2**, dosages above 200 µg/mL initially increased viability but then showed a decreasing trend. Based on the MTT assay results, 100 and 200 µg/mL LAA were selected as the effective concentrations for subsequent experiments.

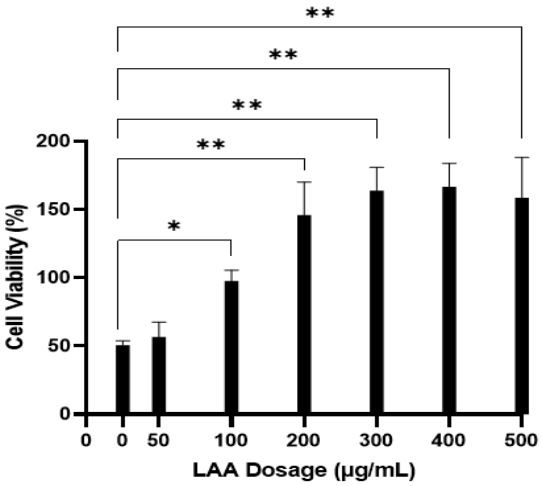

Figure 2 Cell viability (%) following the addition of LAA at various concentrations (µg/mL) was assessed using the MTT assay. The results were obtained after 48 h of incubation with 2% HS. Statistical analysis with ANOVA showed significant differences ($^{*}p < 0.05$; $^{**}p < 0.01$). Data are presented as mean ± standard deviation (SD) in percentage.

In this study, we found that culture media supplemented with LAA maintained the viability of AT-MSC. The optimal concentrations were 100 and 200 µg/mL. Increasing the LAA concentration led to an increase in cell viability; however, although the increase was statistically significant, a decrease in viability was observed at 500 µg/mL. Fujisawa *et al.* [12] reported that LAA phosphate 0.1, 1.0 and 3.0 mM promoted MSCs proliferation at P4, with all concentrations showing a similar degree of enhancement.

LAA possesses antioxidant properties that protect stem cells from oxidative stress and damage. By scavenging ROS, LAA reduces oxidative damage, which is crucial for maintaining stem cell viability. [13] A previous study reported that low-dose LAA treatment (400 µm) did not affect the sphere-forming ability of neural stem/progenitor cells (NSPCs). However, when cells were exposed to high dose LAA (2 - 5 mM), they became loosely connected, began to adhere to the plate, and showed a reduction in both cell number and size. High dose LAA demonstrated toxic effect on stem cells by depleting intracellular glutathione (GSH), inducing oxidative stress, and causing DNA damage [14]. In this study, we selected LAA dose of 100 and 200 µg/mL for further experiments, which are equivalent to approximately 500 - 600 µm and categorized as low dose LAA. These concentrations were chosen as the lowest effective dose that showed measureable effect. We concluded that increasing the LAA dose beyond this range did not further enhance cell expansion but instead reduced the survival potential of the cells.

Effect of LAA on morphology of AT-MSCs

The morphology of AT-MSCs in culture after LAA treatment is shown in **Figures 3(A) - 3(F)**. After 3 days of LAA treatment, the cells exhibited a fibroblast-like adherent morphology. In contrast, the control group showed round-shaped cells that appeared larger than those in the LAA-treated groups. The 200 µg/mL LAA treatment resulted in a pronounced increase in the AT-MSC population, as observed in **Figures 3(C**) and **3(F)**. Both passages exhibited similar morphological characteristics.

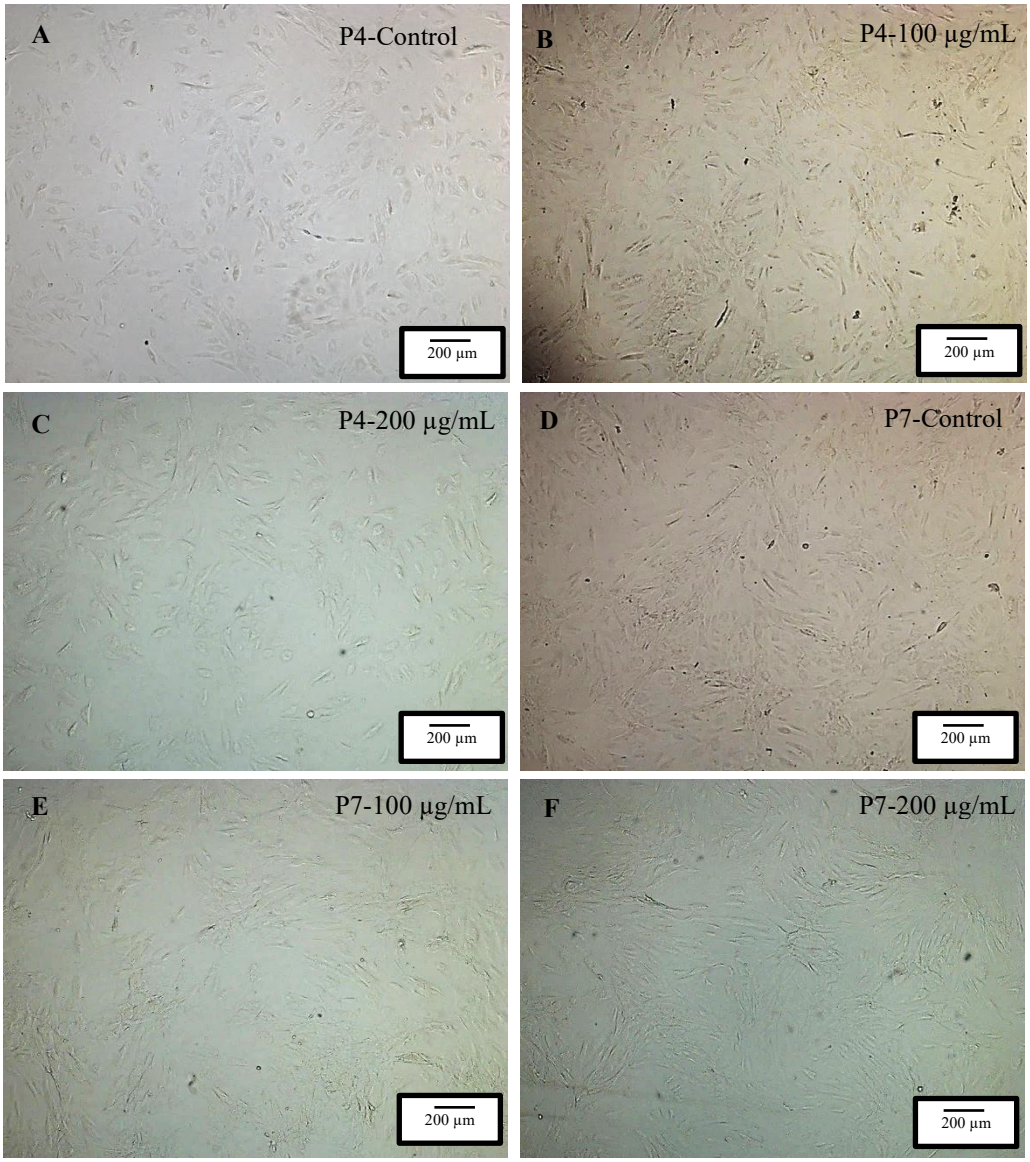

Figure 3 (A) - (F) Representative images showing fibroblast-like morphology of AT-MSCs following treatment with LAA at different concentrations (Control non LAA, 100 and 200 µg/mL LAA) from P4 and P7; magnification 4X, scale bar: 200 µm.

Effect of LAA concentration on proliferation and viability of AT-MSCs

The proliferation and viability of AT-MSCs in each group are shown in **Figure 4**. To evaluate the effects of LAA at concentrations of 100 and 200 µg/mL on AT-MSC at P4 and P7 compared to the control, the initial seeding densities were kept consistence across groups: 375×10^3 cells/well for P4 and 125×10^3 cells/well for P7. Total cell numbers were counted after 7 days of culture at both passages. LAA demonstrated a stimulatory effect on cell growth, with a significant ($p < 0.01$) observed at 200 µg/mL in P4 compared to P7, while no significant difference was found between the 2 tested concentrations (**Figure 4(A)**).

Several studies have shown that LAA induce various cellular responses, although the mechanisms remain unclear across different cell types. In this study, LAA treatment increased cell proliferation after 7 days, particularly in the P7 group, as indicated by the cell ratio. In contrast, the P4 group showed less pronounced differences. The limited effect of LAA at P4 may be due to the treatment starting at P3, suggesting that the proliferation observed at P7 reflects the cumulative effect of LAA from P3 to P7. The significant difference between P4 and P7 indicates that the proliferation of

AT-MSCs was optimally enhanced after 7 days of LAA treatment, especially at the 200 µg/mL concentration.

The p53 pathway is also reported to play an important role in cell cycle regulation. Suppression of p53 has been shown to promote cell proliferation by preventing cell cycle arrest. Previous studies using p53 knock-out mice demonstrated that the proliferation rate of MSCs was significantly higher compared to that in wild-type mice [15-17]. In this study, 200 µg/mL LAA dose was identified as the optimal concentration for enhancing AT-MSCs proliferation, demonstrating dose-dependent effect that was greater than that observed in the study by Zhang *et al*. Both 200 and 100 µg/mL LAA doses showed no statistically significant different in term of safety and exhibited no cytotoxic effects on AT-MSCs, thereby classifying them as low dose LAA treatments [15].

Viable and non-viable cells were counted to determine cell viability in each group at P4 and P7. Cell viability remained stable from P4 to P7 across all groups; however, a significant difference was observed between the P4 groups and the untreated group ($p < 0.01$) (**Figure 4(B)**).

This study demonstrated that LAA helped maintain the viability of AT-MSCs, although a slight decrease was observed in the P4 group after LAA treatment. There was no significant difference in viability between the 2 LAA doses. Despite the observed decrease in P4, overall viability remained stable, with values above 90% following LAA treatment up to a dose of 200 µg/mL. This supports its classification as low-dose treatment (equivalent to 500 - 600 µm) [15].

Some studies have reported that a cell viability in the range of 80% - 95% is considered indicative of good viability [18]. A decrease in viability has been reported at higher concentration of LAA (750 - 1,000 µm) [19]. LAA is also known to function as an anti-proliferative agent. Valenti *et al.* [20] reported that, in human osteoblast cells, higher doses of LAA (750 - 1,000 µm) significantly increased p21 gene expression, leading to cell cycle arrest [21]. This finding aligns with the observed decline in cell viability at those concentrations. In the present experiment, LAA treatment may have continued to enhance the viability of AT- MSCs at doses higher than 200 µg/mL, potentially up to 300 µg/mL. However, doses beyond this range may result in diminished viability due to the onset of cytotoxic effects.

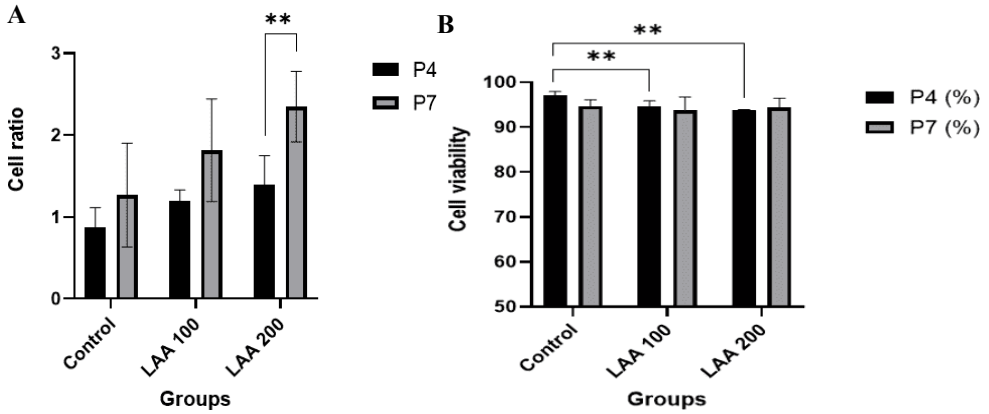

Figure 4 (A) Cell ratio showing the comparison of total cells after 7 days treatment relative to the initial cell number, between P4 (black) and P7 (grey). Data were normally distributed and homogenous. ANOVA test for both P4 and P7 showed no significant different ($p > 0.05$). Paired *t*-test and Wilcoxon tests across all treatments in both passages were not significant ($p > 0.05$), except in the LAA 200 µg/mL group (**$p < 0.01$). (B) Cell viability (%) in P4 (black) and P7 (grey). ANOVA test in P4 revealed significant difference (**$p < 0.01$), whereas no significant different was found in P7 ($p > 0.05$). Paired *t*-tests between P4 and P7 for each treatment group were not significant ($p > 0.05$). Data are presented as mean ± SD in percentage.

Effect of LAA concentration on characterization of AT-MSCs in specific different marker

The characterization of AT-MSC was confirm by flow cytometry. The results showed that AT-MSC from P4 to P7 were positive for CD90, CD73, and CD105, while exhibiting very low expression of hematopoietic stem cells (HSC) surface antigens. There were no significant differences in the expression of CD90, CD73, and HSC markers between groups (P4 vs P7; control vs LAA-treated groups) ($p > 0.05$). However, CD105 expression was significantly higher in the 100 µg/mL LAA-treated group compared to the untreated group ($p < 0.05$) (**Figures 5(D) - 5(G)**).

The characterization of AT-MSCs were determined by the expression of specific surface markers, including CD73, CD90, and CD105 [13]. The percentage expression of CD90 and CD73 ranged from 60% to 100%, while CD105 expression was below 30% at both P4 and P7. In contrast, hematopoietic stem cells marker (CD34/CD45/HLA-DR-PE A) were very low (< 1%), indicating negative expression (**Figures 5(A) - 5(C)**).

Stem-cell related surface markers are typically more highly expressed at P3, while hematopoietic markers remain negative [22]. In this study, a significant increase in CD105 expression was observed at P4 following treatment with 100 µg/mL LAA, indicating a slight improvement in cell quality. At higher passages (P7), although cells continue to proliferate, the accumulation of senescent cells increase. It was observed that LAA enhanced CD105 expression at lower passage; however, this effect diminished at higher passages. Despite the observed trend, the difference was not statistically significant. Further research is needed to confirm the role of LAA supplementation in regulating the CD105 signaling pathway in AT-MSCs, as well as its involvement in other aging-related pathways. Additionally, identifying the most effective LAA dose for AT-MSC culture requires further investigation. Overall, this study showed that CD105 expression was generally low; although an enhancement was observed following LAA treatment, CD105 expression remained inconsistent.

CD105 is considered an important marker for MSC; however, its expression varies depending on MSC source, culture duration, culture conditions and differentiation state [23,24]. Changes in endoglin (CD 105) expression have been observed in later passages, likely due to the tendency of human AT-MSCs to undergo osteogenic differentiation [22]. Some studies suggest that CD105 expression is related to differentiation potential, $CD105^-$ AT-MSC appear more prone to osteogenic differentiation, whereas $CD105^+$ AT-MSC exhibit greater chondrogenic potential. However, other studies have reported contradictory findings. A recent study reported that CD105 expression did not significantly affect the stemness or differentiation capacity of AT-MSCs [25].

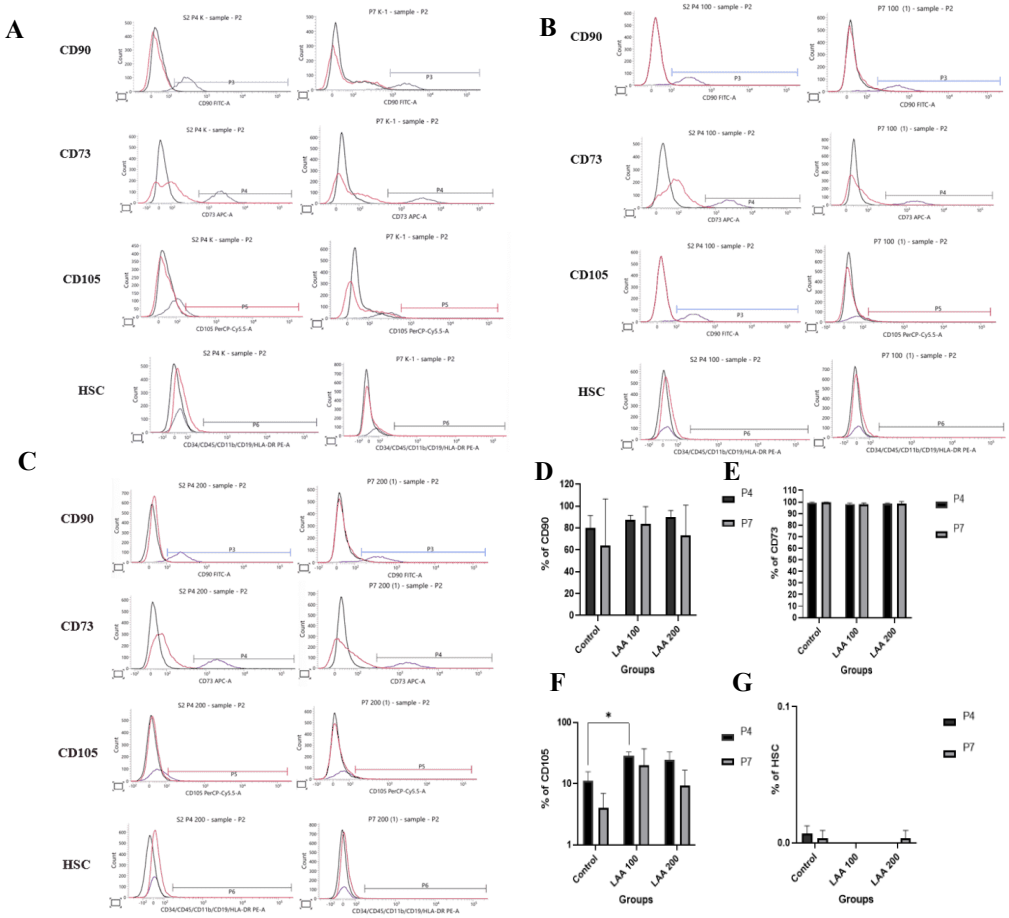

Figure 5 (A) - (C) Representative overlaid histograms for phenotypic characterization of AT-MSCs in the control, 100 µg/mL LAA, and 200 µg/mL LAA groups, as assessed by flow cytometry (D) - (G) Molecular markers expression of AT-MSC at P4 (black) and P7 (grey) indicating characteristic surface markers. ANOVA tests for both P4 and P7 showed no significant different (*$p > 0.05$), except for CD105 expression at P4 in the dose 100 µg/mL (*$p < 0.05$). Paired t-test and Wilcoxon test across all treatment and passage were not significant ($p > 0.05$). Data were obtained from gated cells population and are presented as mean ± SD of the percentage of positive expression.

Effect of LAA concentration on differentiation ability of AT-MSCs

The differentiation ability of AT-MSCs is depicted in **Figure 6**. The results show that AT-MSCs differentiated into chondrocytes (A) - (B) and osteocytes (C) - (D) after 30 days of incubation, and into adipocytes (E) - (F) after 14 days of incubation. **Figures 6(A) - 6(C)** represent AT-MSCs at P4 while **Figures 6(D) - 6(F)** represent P7. This finding indicates that AT-MSCs retained the ability to differentiate into chondrocytes, osteocytes and adipocytes from P4 to P7, with no apparent differences in morphology across the experimental groups. The groups treated with 100 and 200 µg/mL LAA showed similar differentiation profiles compared to the control group.

The differentiation capacity of LAA supplemented AT-MSCs, appeared histologically similar. However, this experiment showed remained ability of AT-MSCs to differentiate into 3 cells lineages (chondrocytes, osteocytes and adipocytes) even after LAA supplementation. The generally low expression of CD105 observed in this study may suggest a tendency toward osteogenic differentiation. Nonetheless, the absence of quantification of the overall percentage of differentiated AT-MSCs limits the ability to draw definitive conclusions regarding their osteogenic potential.

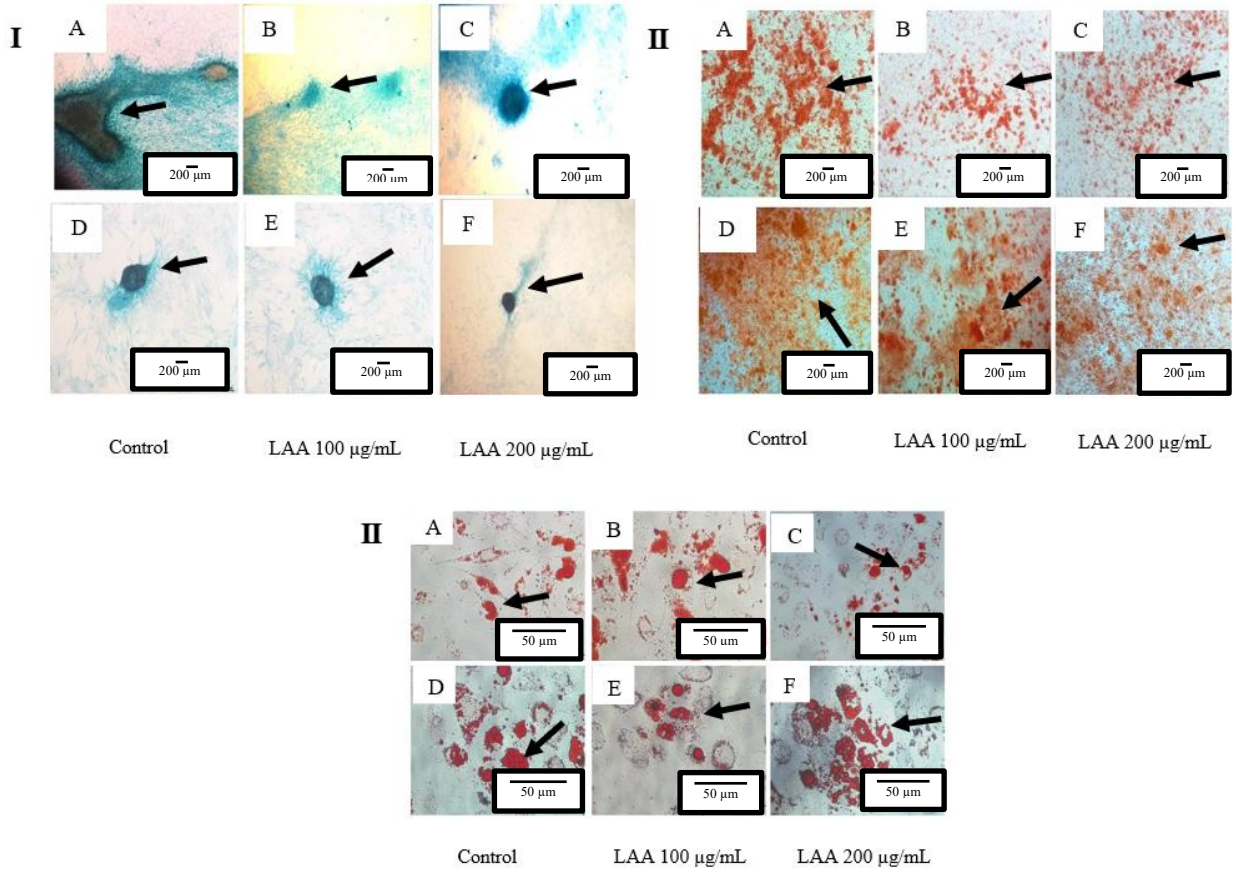

Figure 6 Representative images of the multilineage differentiation ability of expanded AT-MSCs. Cell morphology and histological staining in 2-dimentional culture under different induction conditions: (I) Chondrogenic differentiation stained with Alcian Blue; (II) Osteogenic differentiation stained with Alizarin Red; (III) Adipogenic differentiation stained with Oil Red O; Panel A-B represent P4, C-D represent P7. Image was captured at 4X magnification. Scale bars: 200 and 50 µm).

LAA support chondrogenesis by promoting collagen synthesis and stabilizing the extracellular matrix, which is essential for cartilage formation. It enhances the secretion of type II collagen, a major component of cartilage and upregulates key chondrogenic markers such as aggrecan, Sox9, and collagen type II. L-ascorbic acid functions as a cofactor for enzymes involved in the hydroxylation of proline and lysine residues during collagen synthesis, thereby contributing to the structural integrity of the extracellular matrix required for proper chondrogenic differentiation.

LAA also play a key role in promoting the differentiation of AT-MSCs into osteocytes by enhancing osteogenic differentiation and facilitating extracellular matrix (ECM) mineralization [27]. It increases the expression of critical osteogenic markers, including alkaline phosphatase (ALP), osteopontin, osteocalcin, and runt-related transcription factor 2 (Runx2). Furthermore, LAA modulates several signaling pathways essential for osteogenesis: it upregulates Runx2, enhances the Wnt/β-catenin signaling by increasing the β-catenin levels, and influences the transforming growth factor-beta/TGF-β/Smad pathway [28].

The role of LAA supplementation in AT-MSCs differentiation into chondrocytes involve its influence on bone and cartilage development. Previous studies have reported that ascorbic acid deficiency can lead to reduced chondrocytes proliferation and impaired matrix synthesis [13]. Chang *et al.* [29] demonstrated that LAA exerts contradictory effect: it stimulates the expression of collagens and proteoglycans yet inhibits chondrocytes differentiation under condition of oxidative stress. The effects of LAA on MSC differentiation are mediated through multiple regulatory

pathways, including the protein kinase C/nuclear factor erythroid 2 (PKC/Nrf2) pathway and the c-Jun N-terminal kinase/activator protein-1 (JNK/AP1) signaling pathway.

While LAA primarily stimulates collagen synthesis, crucial for maintaining the structural integrity of ECM, it also provides a supportive environment for adipocyte maturation. LAA influences the expression of adipogenic markers and transcription factors, such as peroxisome proliferator-activated receptor gamma (PPARγ), which play a central role in adipocyte differentiation. In addition, LAA increases the expression of PPARγ and enhancer-binding protein α (EBPα), both of which are essential for the early stages of adipogenic commitment. A key effect of LAA is its ability to enhance the accumulation of intracellular lipids, a hallmark of adipocyte differentiation [28].

The ECM is composed of collagen, proteoglicans/glycosaminoglicans, elastin, fibronectin, laminins, and various other glycoproteins. This matrix plays a crucial role in cell adhesion and is present in all tissues, including the skin. The ECM is essential in maintaining the structural integrity and function of the skin [10]. Fujisawa *et al.* [12] reported that LAA treatment could serve as a substitute for collagen-coated culture conditions to enhance cell proliferation. LAA is a critical cofactor for hydroxylase, which are involved in the posttranslational modification of collagen molecules [30]. Supplementing culture media with LAA has been shown to enhance collagen synthesis through multiple mechanisms. In this study, LAA treatment was administrated starting at P3. However, given its role in promoting collagen synthesis, LAA supplementation may be more effective if introduced at passages 0 (P0), to enhance AT-MSCs attachment to plastic surface and support greater proliferation. Supplemented culture media with LAA has been shown to enhance collagen synthesis through various mechanisms. These findings highlight the potential clinical applications of AT-MSCs and their LAA supplemented secretome products in regenerative medicine, particularly for addressing skin aging.

Effect of LAA concentration on AT-MSC senescence

The effect of LAA treatment on AT-MSCs senescent was measured using the SA-β-Gal assay, as shown in **Figure 5**. A decrease in the percentage of senescent cells was observed after treatment with 100 and 200 µg/mL LAA, with the lowest count significantly at 100 µg/mL compared to the control. However, senescent cell was not significantly reduced across passages. Following the SA-β-Gal assay, the presence of blue-stained cells indicated a positive result for cellular senescence (**Figures 7(A) - 7(C)**). A significant different was observed ($p < 0.05$) (**Figure 7(D)**), with LAA treatment appearing to reduce the number of blue stained cells, particularly in the 100 µg/mL LAA group.

LAA is a natural antioxidant that can scavenge ROS, thereby influencing the AKT and mTOR signaling pathways, both of which are associated with stem cell aging [7]. This study evaluated the effect of specific LAA doses on the number of senescent cells. Yang *et al.* [7] reported that in MSCs induced by d-galactose, an increase in cellular senescence, ROS level, and activation of the AKT/mTOR signaling pathway was observed, highlighting the interplay between oxidative stress and senescence. To inhibit this pathway, ROS also plays a role in inactivating protein tyrosine phosphatase 1B (PTP1B) and protein phosphatase 2A (PP2A), which are key regulators that suppress AKT/mTOR activity [31]. In this context, the addition of LAA led to AKT/mTOR inhibition, and the anti-senescent effect of LAA was clearly demonstrated in this study.

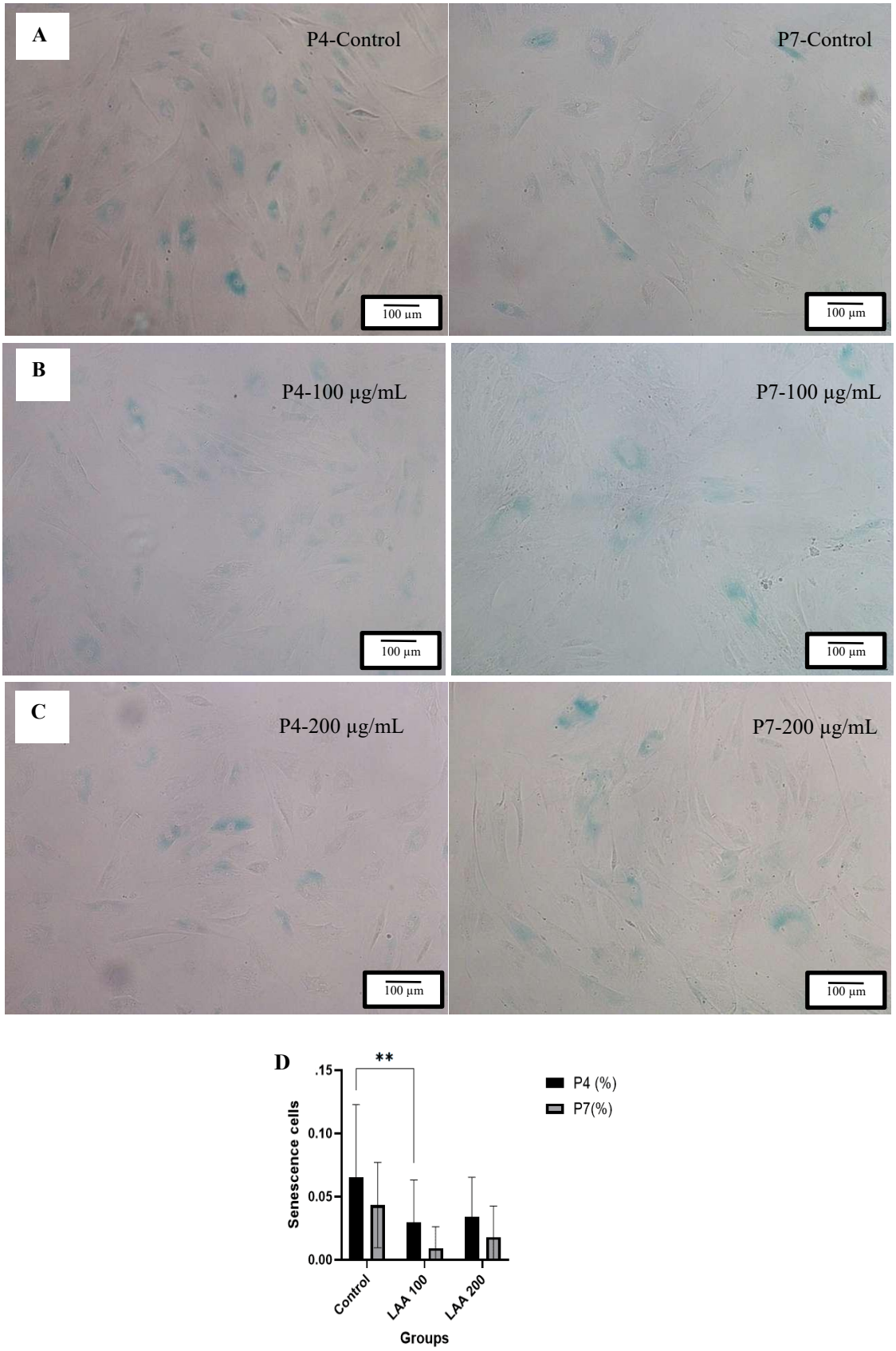

Figure 7 (A) - (C) SA-β-Gal assay of AT-MSCs showing blue-stained senescent cells; magnification 40X, scale bar: 100 µm (D) Percentage of senescent cells in culture as determined by SA-β-Gal assay, comparing P4 (black bar) and P7 (grey bar). ANOVA test revealed a significant different (*$p < 0.05$). Data are presented as mean ± SD in percentage.

In this study, LAA supplementation at 200 µg/mL significantly increase AT-MSCs proliferation, while the 100 µg/mL dose significantly reduced the number of senescent cells. These findings suggest that the optimal LAA dose for in vitro supplementation requires further optimization, particularly for large-scale culture expansion [32,33]. Although LAA has been reported to maintain the viability and differentiation capacity while reducing cellular senescent over extended passages, maintaining the stability and efficacy of LAA during prolonged culture remains a challenge for clinical applications.

Conclusions

In summary, we have evaluated the effects of LAA at doses 100 and 200 µg/mL LAA on AT- MSC. These doses were found to help maintain cell morphology, enhance proliferation and viability, and support differentiation potential. Throughout the passages, AT-MSC characteristics remained relatively consistent; however, the expression of CD 105 did not align with typical MSCs profile. Notably, treatment with 100 µg/mL LAA significantly reduced the number of AT-MSC senescent cells during early passages. These findings suggest that LAA may hold potential for use in regenerative medicine, particularly in anti-aging strategies. However, further research, especially long term and in-vivo studies is necessary to validate these results and to better elucidate the underlying mechanisms before any clinical application can be considered.

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Declaration of Generative AI in Scientific Writing

The authors acknowledge the use of generative AI tools (e.g., ChatGPT by OpenAI) in the preparation of this manuscript, specifically for language editing and grammar correction. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

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Komang Ardi Wahyuningsih: Conceptualization, Methodology, Supervision, Validation, Revision, Funding acquisition, and Writing – original draft. **IGede Eka Wiratnaya**: Data curation, Formal analysis, Investigation, Validation and Visualization. **IWayan Weta**: Data curation, Formal analysis, Investigation, Validation and Visualization. **IGede Raka Widiana**: Data curation, Formal analysis, Investigation, Validation and Visualization. **Wimpie Pangkahila**: Data curation, Formal analysis, Investigation, Validation and Visualization. **Ida Ayu Ika Wahyuniari**: Data curation, Formal analysis, Investigation, Validation and Visualization. **IMade Muliarta**: Data curation, Formal analysis, Investigation, Validation and Visualization. **Veronika Maria Sidharta**: Methodology, Project administration, Resources, Supervision, Validation, and Writing – original draft. **Retnaningtyas Siska Dianty**: Conceptualization, Software, and Writing – review & editing.

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